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Misc Tr
464

Investigations with fluorescence-labeled antibodies. IV. Labeling of antibodies with sulfochlorides of fluorescent dyes.

by H. Uehleke.

Translated from Schweiz. Z. Path. Bakt. 22: 724-729 (1959) by the Technical Library, Technical Information Division.

The isocyanate method acquired great significance from customary chemical reactions, in which protein substances are combined with other molecules, by the idea (Coons, 1941, 1950) of linking fluorescein to proteins and visualizing reactions between antigens and antibodies directly in this manner.

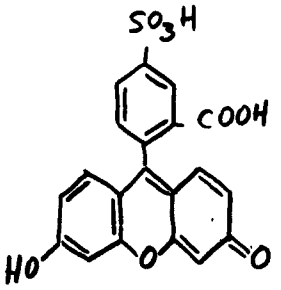
Other dyes have also been utilized in this method: β-anthryliso-cyanate by Creech and Jones (1941), rhodamine isocyanate by Silverstein (1957) and tetramethyl-rhodamine by Hiramoto et al. (1958).

Due to the complicated preparation and great sensitivity of iso-cyanates, the search for other methods was continued. Hess and Pearse (1959) recently discovered an original technique in reactions between proteins and halogenated triazines linked to fluorescent dyes. The fairly stable isothiocyanate of fluorescein was recommended by Marshall et al. (1958).

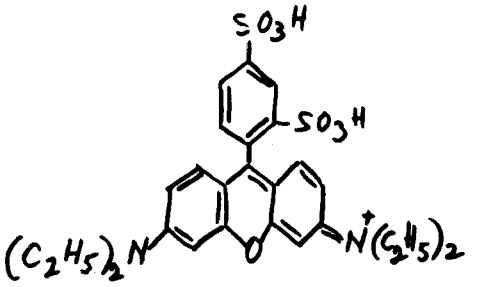
In 1954 Clayton experimented with the sulfochloride of 1-dimethyl-amino-naphtalene-5-sulfonic acid, the linkage of which to proteins was studied by Weber (1952) and successfully applied by Laurence (1957), Mayersbach (1958), Redetzki (1958), Petuely (1958), Wolochow (1959) and others.

We have tried to link different fluorescent dyes with sulfo groups via the appropriate sulfochlorides to proteins (Uehleke, 1958a and b). In this connection, derivatives of xanthene, acridine and pyrene were examined in greater detail.

Xanthene: Condensation of 4-sulfophthalic acid with resorcinol did lead to a product amenable to chlorination and linkage to proteins. However, we have been unable to produce 4-sulfophthalic acid in pure form, indicating that our sulfofluorescein also represented a mixture.



sulfofluorescein

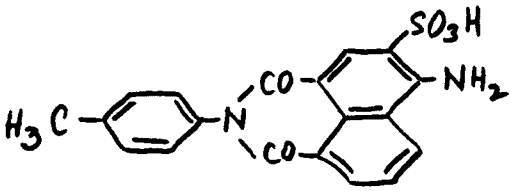


sulforhodamine B

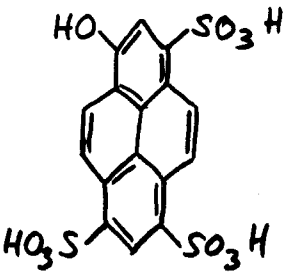
Commercial sulforhodamine B, on the other hand, is readily chlorinated with PCl_5 and attached to proteins. Its orange-red fluorescence in UV light contrasts superbly with the unavoidable autofluorescence of numerous cells and tissues. Sulforhodamine had been reported independently by Chadwick et al. (1958) as a suitable substance.

Acridine: Acridine orange yields some decomposition products upon direct sulfurization, from which sulfo-acridine orange is not readily isolated in pure form. Such impurities unfortunately impede subsequent chlorination.

Other fluorescent dyes with sulfo groups are available commercially. Among these, brilliant sulfoflavin (FF) and 3-hydroxy-5,8,10-pyrene-trisulfonic acid were marked by particularly strong fluorescence.



brilliant sulfoflavin (FF)



3-hydroxy-5,8,10-pyrene-trisulfonic acid

Unfortunately brilliant sulfoflavin does not react as smoothly with PCl_5 under customary conditions, and heating probably produces degradation products. Pyrene-trisulfonic acid also fails to react with PCl_5 as favorably as sulforhodamine; it requires dehydration and subsequent heating with PCl_5.

In treating the sodium salt of a sulfonic acid with PCl_5, the presence of moisture (crystal water), the temperature, the solvent and other factors decide whether the reaction continues with the resultant $POCl_3$:

$$\begin{aligned} R\text{-}SO_3Na + PCl_5 &\longrightarrow R\text{-}SO_2Cl + POCl_3 + NaCl \\ 2R\text{-}SO_3Na + POCl_3 &\longrightarrow 2R\text{-}SO_2Cl + NaPO_3 + NaCl \\ \hline 3R\text{-}SO_3Na + PCl_5 &\longrightarrow 3R\text{-}SO_2Cl + NaPO_3 + 2NaCl \end{aligned}$$

Sulfochlorides have good solubility in such organic solvents as ether and acetone, permitting their separation from unreacted dye.

These sulfochlorides may now react with primary, secondary amines and hydroxyl groups in the protein molecule:

$$R\text{-}SO_2Cl + H_2N\text{-}R' \longrightarrow R\text{-}SO_2\text{-}NH\text{-}R' + HCl$$

$$R\text{-}SO_2Cl + 2HNR'R'' \longrightarrow R\text{-}SO_2\text{-}N\begin{matrix} \nearrow R' \\ \searrow R'' \end{matrix} + HNR'R'' + HCl$$

If the proposed excess sulfochloride is treated with primary amine, secondary sulfamines may result:

$$2R\text{-}SO_2Cl + 3H_2NR' \longrightarrow (R\text{-}SO_2)_2NR' + 2H_2NR' + HCl$$

Sulfonic acid esters with alcohols and phenols are readily formed at ordinary or slightly raised temperatures in the presence of acid-binding substances:

$$R\text{-}SO_2Cl + HOR' \longrightarrow R\text{-}SO_3R' + HCl$$

Few dye molecules combine with a protein molecule at a molecular weight of 50 to 150,000 for globulins. Presumably only a few spatially extended amino and carboxyl groups are reactive. The others are partly related via hydrogen links or else substitution is made impossible sterically, i.e., due to spatial impediments.

We let the solution of the appropriate sulfochloride (dissolved in acetone, for example) drip very slowly into the protein solution which is held at pH 8.5 - 9.0 with tris-buffer. Tri(hydroxymethyl)-aminomethane has a maximal buffering capacity at about pH 9 and for this reason is more suitable than a CO_2/HCO_3 buffer (pK H_2CO_3 = 6.35). The entire procedure is carried out in the cooler at +2°C and the solution is left standing for a few hours. Next, dialysis is performed against m/100 phosphate buffer, pH 7.4. Greatly diluted protein solutions may now be recompressed rapidly and carefully by ultrafiltration.

In order to further reduce non-specific staining with labeled antibodies treated in this manner, one or more of the customary purification methods may follow. Adsorption on liver powder, homologous material or activated charcoal has been most effective.

Paper electrophoresis is very well suited for testing of complete attachment to proteins and for removal of excess dye. Essentially, the rate of migration depends on the molecular size and the charge. Proteins coupled to pyrene-trisulfonic acid migrated more rapidly than untreated ones, since the free sulfo groups (pK $<$ 2) are much more strongly dissociated than carboxyl groups (pK 4.5 to 6) or amino groups (pK 6-8) of the proteins. However, when hydroxypyrene-trisulfonic acid was heated with an excess of PCl_5, the conjugates also slowed down. Evidently the result is a meshing of protein molecules.

We synthesized pyrene-di- and mono-sulfonic acid in order to create conditions of greater clarity. These acids and their sulfochlorides show excellent fluorescence. Unfortunately they lose nearly all of their fluorescence upon linkage to proteins. Perhaps this may be prevented by further substitution with hydroxyl or amino groups.

The demand for a prepared or readily produced fluorescent dye which is adequately stable in its reactive form, attaches itself securely to proteins and does not decolorize under UV illumination is met among sulfo dyes with tolerable satisfaction only by 1-dimethyl-naphtalene-5-sulfonic acid and sulforhodamine B.

Endeavors to give the practising serologist, bacteriologist and histologist a simple fluorescence technique should justify a search for additional dyes and methods.

Discussion

F. Petuely: The substances used by the author for labeling of antibodies, with the exception of 1-dimethylamino-naphtalene-sulfonic acid-5 used by Mayerbach for several years, have several functional groups in the molecule. This results in linkage of several protein molecules to one dye molecule upon attachment to immune globulins,

producing high-molecular products with poor solubility and possible precipitation from the solution. In my opinion, a dye used for labeling of antibodies should have only one functional group. Even when only one protein molecule is attached to a substance with several functional groups, the presence of water causes hydrochloric acid to be separated from the unoccupied functional groups, i.e., the sulfo groups, necessitating the use of a very strong buffer. I should like to refer to my remarks in connection with the discussion of Coons' presentation. Fluorescent dyes with several sulfo groups may also act as dyes in protein conjugation (as in the case of fluorescein) and thus lead to non-specific stains.